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Analysis of organic acids and total acidity in alfalfa (*Medicago sativa* L.) collected from different locations in the Republic of North Macedonia

Valentina Butleska Gjoroska[1]*, Marija Krstik[2], Lenka Cvetanovska[2] and Liljana Koleva Gudeva[1]

[1]Faculty of Agriculture, Goce Delcev University - Stip, Krste Misirkov Str., No 10-A, 2000 Stip, Republic of North Macedonia.
[2]Faculty of Natural Science and Mathematics, Arhimedova Str., No 3, 1000 Skopje, Ss. Cyril and Methodius University of Skopje, Republic of North Macedonia.

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ABSTRACT

The role of alfalfa (*Medicago sativa* L.) in the development of agricultural production and intensification of forage production is due to the high potential of biomass on the one hand anti-oxidative and nutritional value on the other hand. Modern techniques have been used for analysing of organics acids. Organic acids are formed during cell respiration as products of total dissemination of carbohydrates and generally serve as starting substances for the biosynthesis of different compounds such as fatty acids and fats, amino acids, vitamins, coenzymes, pigments and other substances. Organic acids are key metabolites in plants, and their amounts depend on the intensity of cell respiration. The accumulation of some organic acids in plants is closely related to the intensity of enzymatic reactions as well as to various environmental factors. On the cellular level, the metabolism of organic acids is of high importance for several biochemical pathways, such as the cycle of tricarboxylic acids and the glyoxalate cycle. Organic acids that are synthesised in these cycles are used by plants as precursors in the biosynthesis of other organic compounds. Significant differences are found in the production of organics acids between the locations in Ovche Pole region on the one hand and Skopje and Tetovo regions on the other hand. We investigated various organic acids (citric, acetic, wine, malic and lactic acid) and the total acidity in alfalfa (*Medicago sativa* L.) from three different regions of the Republic of North Macedonia, in 19 different locations and three harvests.

Keywords: Acetic acid, citric acid, lactic acid, malic acid, wine acid, alfalfa.

*Corresponding author. E-mail: tina_valentina2@yahoo.com.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial leguminous fodder crop and the most important forage plant. It is one of the oldest forage plants and, because it is perennial, shows high yields with a high quality. It can regenerate rapidly and provides five to six harvests during a vegetation season. It produces high-quality fodder for all types of domestic animals (Ivanovski, 2000).

The great interest in cultivating this species is based on its high nutritional value as well as the high fibre digestibility and content (Julier et al., 2000). Alfalfa has a high concentration of protein (Dinic et al., 2005) with a favourable amino acid composition, resulting in a high biological value. It also contains high amounts of important vitamins, carbohydrates, saponins and mineral elements, especially calcium. In addition, important chemical elements and other active components, essential for the growth and development of animals, are present in alfalfa (Hao et al., 2008). In this sense, alfalfa is a dominant fodder culture and an active dietary culture with high applicability in bio-nutrition (Markovic et al., 2007).

Alfalfa is used as a perennial culture continuously for 4 to 5 years, providing four to five harvests per year. In temperate regions, alfalfa should be harvested every 4 to 6 weeks. Alfalfa plays an important role in crop rotation and provides large quantities of organic matter to the soil, thereby improving the physical, chemical and microbiological properties of the soil as well as the soil structure. As a nitrogen-fixing plant it enriches the soil with readily available nitrogen, which makes alfalfa an excellent pre-culture for numerous cereal, industrial and horticultural crops. Unlike most other fodder legumes, alfalfa is usually grown in pure monocultures, although it can be mixed with other legumes and grasses (Walton, 1983).

In North Macedonia, this crop is grown on an area of 19,000 ha, with an average yield of 6 to 6.5 t/ha (Ilievski, 2013).

In the metabolism of plants, organic acids are formed by the decomposition of carbohydrates, fats and proteins or of amino acids in the cycle of tricarboxylic acids, also known as the Krebs cycle, the citric acid cycle or the citrate cycle. The amount of organic acids in plants depends on the intensity of respiration. Organic acids are formed during the cell respiration in plants as products of the total dissemination of carbohydrates (Benet-Clark, 1993). The aim of the research is to determine the presence of organic acids in the examined culture alfalfa (*M. sativa* L.) at the examined locations in the respective regions of the territory of the Republic of North Macedonia. Depending on the presence of organic acids, it can be determined in which location or region it is more favorable cultivation of alfalfa. The higher the production of organic acids, the more we get better cultivation of culture.

The purpose of the research is to evaluate the value of organic acids as dietary supplements to humans

MATERIALS AND METHODS

Plant material

Alfalfa material (*M. sativa* L.) was collected from three different regions in the territory of the Republic of North Macedonia: the Skopje region, the Ovche Pole region and the Tetovo region, from 19 different locations in three harvests in (Table 1). The material was collected during the vegetative cycle (from June to August) in 2013. In the first, second and third harvest, plants were collected on June 15, July 17 and August 15, respectively. Analysis of organic acids was conducted on dry plant material using the neutralization method.

Procedure for carrying out the analysis

First, a certain amount of plant material was measured, depending on the amount of water it contained or the contents of the acids. We used three samples for each analysis. The sample was dried in a porcelain bowl by adding a 5 mg of quartz sand and 2 to 20 ml of distilled water, macerated to a homogeneous mixture and subsequently quantified by flushing with 50 ml of distilled water in a 250 ml measuring flask. The flask was filled with water to the measuring line, 1 ml of toluene was added to prevent boiling and the acids were extracted over 2 h with uniform mixing. Extraction of the acid fraction was accelerated by adding distilled water to the mass after transfer into the measuring flask to 2/3 of the volume; extraction was carried out in a thermostat or water bath with constant stirring for 20 to 30 min at a temperature of 68 to 70°C.

Extracting with backward cooling can also be used. Subsequently, the mixture was cooled to a normal temperature (22°C), supplemented with water to the mark, and filtered or centrifuged. This resulted in a transparent, poorly opalescent extract containing dissolved acids. We transferred 25 or 50 ml of the filtrate to an Erlenmeyer flask and titrated with 0.1 N NaOH solution in the presence of phenolphthalein until the appearance of a rosy color.

Determination of total acidity

Total (general, titration) acidity was determined by neutralisation using a base solution (NaOH, KOH) at 0.1 N. Acidity was expressed as ml of 0.1 N NaOH used for titration of 100 g of dry plant material; the results were expressed as percentage of total acidity per dry matter (DM) (Kolthoff and Stenger, 1952).

Statistical analysis

The data were analysed (XLSTAT 2014) via one-way variance analysis (ANOVA) to determine the significant differences ($p < 0.05$) between the mean values of the samples. Subsequently, the results were post-hoc analysed using Duncan's multiple ranking test to determine statistically significant differences in the contents of organic acids and in total acidity among the three harvests.

RESULTS AND DISCUSSION

Contents of organic acids and their total acidity in the first, second and third harvest

The results obtained from the determination of organic acids and their total acidity are presented. We investigated the organic acids acetic, citric, lactic, malic and wine acid and determined their total acidity in alfalfa (*M. sativa* L.)

Contents of organic acids in the first harvest

Table 2 shows that in the first harvest, higher concentrations of citric, acetic, wine and lactic acid were found in the Ovche Pole region (Obleshevo). In addition, a higher concentration of malic acid was registered at the Vrutok location in the Tetovo region, in contrast to the Skopje region (Radishani, Glumovo), where the levels of organic acids were lower.

According to Table 3, all investigated organic acids are in a strong positive correlation between themselves (for all it is valid: $r = 1.000$, $p = 0.000 < 0.05$), with the exception of the malic acid. Organic acids are weak insignificant positive correlation (for all it is valid: $r = 0.131$, $p = 0.322 > 0.05$) with malic acid. The amounts of

Table 1. Description of the locations from the examined locations altitude (m) and latitude (°N) and longitude (°E).

Number	Location	Region	Altitude (m)	Latitude (°N)	Longitude (°E)
1.	Bogovinje	Tetovo	531.50	419.236.809	209.168.772
2.	Vrutok	Tetovo	682.41	417.665.300	208.381.550
3.	Dzepciste	Tetovo	474.48	420.331.690	210.001.650
4.	Galate	Tetovo	600.73	418.381.370	208.813.700
5.	Zelino	Tetovo	1605.94	419.006.530	211.175.770
6.	Pechkovo	Tetovo	991.87	417.843.700	208.311.530
7.	Jegunovce	Tetovo	658.34	421.245.655	210.875.064
8.	Avtokomanda	Skopje	246.68	420.006.868	214.536.642
9.	Sopiste	Skopje	1017.16	418.638.490	213.083.500
10.	Drachevo	Skopje	264.41	419.352.675	215.098.515
11.	Saraj	Skopje	424.88	420.017.493	212.815.977
12.	Radishani	Skopje	392.32	420.732.769	214.479.917
13.	Vlae	Skopje	256.07	420.072.938	213.801.924
14.	Glumovo	Skopje	274.74	419.817.742	213.103.747
15.	Cheshinovo	Ovce Pole	294.00	418.735.350	222.905.610
16.	Karbinci	Ovce Pole	342.98	417.882.100	222.622.460
17.	Obleshevo	Ovce Pole	297.63	418.639.320	222.622.460
18.	Lozovo	Ovce Pole	277.86	417.806.752	218.995.629
19.	Mustafino	Ovce Pole	289.18	418.407.190	220.789.350

organic acids highly varied, depending on various factors such as respiration. There is experimental evidence linking the metabolism of organic acids to defence mechanisms to environmental stressors, and stress can result in the formation of organic acids. Similar results have been found by other authors (Fujita et al., 2006). According to a previous study (Lopez-Bucio et al., 2001), there is increasing evidence that organic acid metabolism is associated with responses to environmental stress. Organic acids not only act as intermediates in carbon metabolism, but are also key components in mechanisms that some plants use to cope with nutrient deficiencies, metal pollution and in plant-microbe interactions at the root-soil interphase.

Contents of organic acids in the second harvest

Table 4 shows the contents of organic acids in alfalfa (*M. sativa* L.) in the second harvest.

Based on the results in (Table 4), in the second harvest, the concentration of organic acids, apart from malic acid, was higher in the Skopje region (Radishani). Lower contents of all the tested acids, except for the malic acid, occur in the Tetovo region (Vrutok). Malic acid concentration was lower in the Tetovo region (Galate) and higher concentration in Ovche Pole region (Obleshevo).

From Table 5 it can be seen that all investigated organic acids are in a strong positive correlation between themselves ($r=1.000$, $p= 0.000 <0.05$), except for the malic acid. Organic acids are insignificant weak positive correlation ($r = 0.072$, $p = 0.589> 0.05$) with malic acid.

Contents of organic acids in the third harvest

The results for the third harvest are presented in (Table 6). Higher organic acid levels, were noted in the Karbinci location in the Ovche Pole region, while lower levels were measured in the Tetovo region (Bogovinje, Jegunovce).

From Table 7 it can be seen that all investigated organic acids are in a strong positive correlation between themselves ($r = 1.000$, $p = 0.000 <0.05$) except malic. Acid that is in a weakly significant negative correlation ($r = 0.294$, $p = 0.024 <0.05$). It follows that the malic acid is in a weak negative significant correlation with citric, acetic, wine and lactic acid. The increased or decreased content of organic acids in the above-ground or ground organisms in plants depends on the anatomical-morphological characteristics, as well as the chemical composition of plants, which is genetically determined. Other authors have come to similar knowledge, such as the findings of Karim (2007). Plants are exposed to many stress factors such as drought, high salinity or pathogens, which reduce the yield of cultivated plants or affect the quality of harvested products. Previous studies have found similar results (Karim, 2007).

Total acidity in first, second and third harvest

In the first harvest, total acidity was higher in the Ovche Pole region (Obleshevo) and lower in the Skopje region

Table 2. Contents of organic acids in alfalfa (*Medicago sativa* L.) from the study sites in the territory of the Republic of North Macedonia in the first harvest, expressed in % of dry matter (DM).

Number	Location	Citric acid	Signif. differ.	Malic acid	Signif. differ.	Acetic acid	Signif. differ.	Wine acid	Signif. differ.	Lactic acid	Signif. differ.
1.	Bogovinje	5 ± 0.5	a	6.4 ± 1.9	bcde	4.7 ± 0.5	ab	5.9 ± 0.6	abc	7.1 ± 0.7	ab
2.	Vrutok	7.3 ± 0.9	efg	7.5 ± 1.2	f	6.9 ± 0.8	bd	8.6 ± 1.1	b	10.3 ± 1.3	c
3.	Dzepchishte	5.5 ± 1.5	bcd	5.4 ± 1.0	cdef	5.1 ± 1.4	abc	6.4 ± 1.8	def	7.7 ± 2.1	cde
4.	Galate	5.4 ± 1.5	bcd	6.2 ± 1.5	bcd	5.1 ± 1.4	abc	6.3 ± 1.8	cdef	7.6 ± 2.1	cde
5.	Zelino	6.1 ± 1.7	cdef	5.9 ± 1.9	bcd	5.7 ± 1.6	cdef	7.1 ± 2.0	ab	8.5 ± 2.4	abcd
6.	Pechkovo	5 ± 0.8	a	5 ± 0.5	a	4.6 ± 0.7	ab	5.8 ± 0.9	bcd	7 ± 1.1	ab
7.	Jegunovce	6.6 ± 1.8	abcd	6.4 ± 2.3	bcde	6.2 ± 1.7	bcd	7.7 ± 2.1	a	9.2 ± 2.5	bc
8.	Avtokomanda	4.6 ± 1.0	abc	5.1 ± 1.4	cd	4.3 ± 0.9	ab	5.4 ± 1.1	abc	6.5 ± 1.4	defg
9.	Sopishte	6 ± 0.8	bcde	5.2 ± 1.0	cd	5.6 ± 0.8	cde	7 ± 1.0	bc	8.4 ± 1.2	abcd
10.	Drachevo	5.8 ± 0.7	ab	5.9 ± 1.0	bcd	5.5 ± 0.6	cde	6.8 ± 0.8	def	8.2 ± 0.9	abcd
11.	Saraj	6.7 ± 1.7	abcd	6.2 ± 1.2	bcd	6.3 ± 1.6	bcd	7.8 ± 2.0	a	9.4 ± 2.3	bc
12.	Radishani	4.3 ± 0.3	abc	5 ± 0.4	cd	4.1 ± 0.3	bc	5.1 ± 0.4	abc	6.1 ± 0.5	def
13.	Vlae	5.4 ± 0.9	bcd	5.5 ± 0.7	abcd	5.1 ± 0.8	cde	6.4 ± 1.1	def	7.7 ± 1.3	cde
14.	Glumovo	5.6 ± 1.5	bcd	4.8 ± 0.4	abc	5.3 ± 1.4	cde	6.6 ± 1.8	def	7.9 ± 2.1	cdef
15.	Cheshinovo	4.7 ± 0.3	abc	5,3 ± 0,8	cdef	4.4 ± 0.3	ab	5.5 ± 0.4	abc	6.6 ± 0.5	defg
16.	Karbinci	6.6 ± 1.8	abcd	7.1 ± 1.7	abc	6.2 ± 1.7	bcd	7.7 ± 2.1	a	9.3 ± 2.6	bc
17.	Obleshevo	7.4 ± 1.4	efg	7.4 ± 2.0	f	6.9 ± 1.3	bd	8.7 ± 1.6	b	10.4 ± 1.9	c
18.	Lozovo	5.4 ± 0.5	bcd	6 ± 1.1	bcd	5.1 ± 0.4	cde	6.3 ± 0.6	cdef	7.6 ± 0.7	cde
19.	Mustafino	5.9 ± 1.7	cdef	6.7 ± 1.3	bcde	5.5 ± 1.6	cdef	6.9 ± 2.0	def	8.3 ± 2.4	abcd

The values in each column marked with the same letters do not differ significantly for $p < 0.05$ according to the Duncan test.

Table 3. Partial correlation coefficients for organic acids in relation to measuring points for first harvest.

Control variables			Citric acid	Malic acid	Acetic acid	Wine acid	Lactic acid	Total acidity
Region	Citric acid	Correlation	1	0.131	**1**	**1**	**1**	0.976
		Significance (2-tailed)		0.322	0 **	0 **	0 **	0 **
		df	0	57	57	57	57	57
	Malic acid	Correlation	0.131	1	0.131	0.131	0.131	0.342
		Significance (2-tailed)	0.322		0.322	0.322	0.322	0.008 **
		df	57	0	57	57	57	57

Table 3. Continues.

Acetic acid	Correlation	**1**	0.131	**1**	**1**	**1**	0.976
	Significance (2-tailed)	0 **	0.322		0 **	0 **	0 **
	df	57	57	0	57	57	57
Wine acid	Correlation	**1**	0.131	**1**	1	**1**	0.976
	Significance (2-tailed)	0 **	0.322	0 **		0 **	0 **
	df	57	57	57	0	57	57
Lactic acid	Correlation	**1**	0.131	**1**	**1**	1	0.976
	Significance (2-tailed)	0 **	0.322	0 **	0 **		0 **
	df	57	57	57	57	0	57
Total acidity	Correlation	0.976	0.342	0.976	0.976	0.976	1
	Significance (2-tailed)	0 **	0.008 **	0 **	0 **	0 **	
	df	57	57	57	57	57	0

** Significantly for $p < 0.05$ according to the Duncan test
Correlation strength: 0-0.50 weak; 0.51-0.75 medium and> 0.75 strong.

Table 4. Contents of organic acids in alfalfa (*Medicago sativa* L.) from the study sites in the territory of the Republic of North Macedonia in the second harvest, expressed in % of dry matter (DM).

Number	Location	Citric acid	Signif. differ.	Malic acid	Signif differ.	Acetic acid	Signif. differ.	Wine acid	Signif. differ.	Lactic acid	Signif. differ.
1.	Bogovinje	7.2 ± 1.4	cde	7.5 ± 1.4	abcd	6.7 ± 1.3	bcd	8.4 ± 1.6	de	10.1 ± 1.9	gh
2.	Vrutok	4.5 ± 1.0	ab	5.8 ± 1.5	ab	4.2 ± 0.9	ab	5.2 ± 1.1	cde	6.3 ± 1.4	cde
3.	Dzepchishte	6.5 ± 1.7	bcde	6.7 ± 1.8	cdef	6.1 ± 1.6	bcd	7.6 ± 2.0	abcd	9.1 ± 2.4	ab
4.	Galate	5.2 ± 0.9	efg	4.7 ± 0.4	abc	4.9 ± 0.9	abc	6.1 ± 1.1	bcd	7.3 ± 1.3	cdef
5.	Zelino	6.3 ± 1.1	bcde	6.5 ± 0.9	cdef	5.9 ± 1.0	b	7.4 ± 1.3	abcd	8.9 ± 1.6	ab
6.	Pechkovo	7 ± 3.0	cde	6.6 ± 2.2	cdef	6.5 ± 2.8	bcd	8.2 ± 3.5	de	9.8 ± 4.3	abcd
7.	Jegunovce	5.7 ± 1.4	efg	6.4 ± 1.6	cdef	5.4 ± 1.3	abc	6.7 ± 1.6	bcde	8 ± 2.0	cde
8.	Avtokomanda	4.7 ± 1.0	ab	5.3 ± 0.5	cde	4.4 ± 1.0	ab	5.5 ± 1.2	cde	6.6 ± 1.4	cde
9.	Sopishte	7.3 ± 0.5	cde	7.4 ± 0.1	abcd	6.9 ± 0.5	cdef	8.6 ± 0.6	def	10.3 ± 0.7	gh
10.	Drachevo	6.1 ± 1.8	bcde	6.1 ± 1.4	ab	5.8 ± 1.7	bcd	7.2 ± 2.1	abcd	8.6 ± 2.5	cd

Table 4. Continues.

11.	Saraj	6.4 ± 1.4	bcde	6.9 ± 1.2	cdef	6 ± 1.3	bcd	7.5 ± 1.6	abcd	9 ± 1.9	ab
12.	Radishani	7.8 ± 1.1	a	8.4 ± 0.9	h	7.3 ± 1.1	cdef	9.1 ± 1.3	h	11 ± 1.6	h
13.	Vlae	7.4 ± 0.6	cde	7.6 ± 0.9	abcd	6.9 ± 0.6	cdef	8.7 ± 0.7	def	10.4 ± 0.8	gh
14.	Glumovo	6 ± 1.4	bcde	6.2 ± 1.3	ab	5.6 ± 1.3	b	7 ± 1.6	cdef	8.4 ± 1.9	cd
15.	Cheshinovo	4.8 ± 0.4	abc	5.1 ± 0.5	abc	4.5 ± 0.4	ab	5.6 ± 0.5	cde	6.8 ± 0.6	cdef
16.	Karbinci	6.9 ± 1.2	cdef	5.9 ± 2.2	ab	6.5 ± 1.2	bcd	8.1 ± 1.4	de	9.8 ± 1.7	abcd
17.	Obleshevo	6.4 ± 1.7	bcde	11.3 ± 7.4	g	6 ± 1.5	cde	7.5 ± 1.9	abcd	9 ± 2.3	ab
18.	Lozovo	7.3 ± 1.6	cde	7.8 ± 1.6	abcd	6.8 ± 1.5	bcd	8.5 ± 1.8	def	10.3 ± 2.2	gh
19.	Mustafino	6.3 ± 1.0	bcde	5.9 ± 1.4	ab	5.9 ± 0.9	cde	7.4 ± 1.1	abcd	8.9 ± 1.4	ab

The values in each column marked with the same letters do not differ significantly for $p < 0.05$ according to the Duncan test.

Table 5. Partial correlation coefficients for organic acids in relation to measuring points for second harvest.

Control variables			Citric acid	Malic acid	Acetic acid	Wine acid	Lactic acid	Total acidity
Region	Citric acid	Correlation	**1**	0.072	**1**	**1**	**1**	0.952
		Significance (2-tailed)		0.589	0	0	0	0
			**		**	**		**
		df	0	57	57	57	57	57
	Malic acid	Correlation	0.072	1	0.072	0.072	0.072	0.374
		Significance (2-tailed)	0.589		0.589	0.589	0.589	0.003
								**
		df	57	0	57	57	57	57
	Acetic acid	Correlation	**1**	0.072	1	**1**	**1**	0.952
		Significance (2-tailed)	0	0.589		0	0	0
			**			**	**	
		df	57	57	0	57	57	57
	Wine acid	Correlation	**1**	0.072	**1**	1	**1**	0.952
		Significance (2-tailed)	0	0.589	0		0	0
			**		**		**	**
		df	57	57	57	0	57	57
	Lactic acid	Correlation	**1**	0.072	**1**	**1**	1	0.952
		Significance (2-tailed)	0	0.589	0	0		0
			**		**	**		**
		df	57	57	57	57	0	57

Table 5. Continues.

Total acidity	Correlation	0.952	0.374	0.952	0.952	0.952	1
	Significance (2-tailed)	0 **	0.003 **	0 **	0 **	0 **	
	df	57	57	57	57	57	0

** Significantly for p <0.05 according to the Duncan test
Correlation strength: 0-0.50 weak; 0.51-0.75 medium and> 0.75 strong.

Table 6. Contents of organic acids in alfalfa (*Medicago sativa* L.) from the study sites in the territory of the Republic of North Macedonia in the third harvest, expressed in % of DM.

Number	Location	Citric acid	Signif. differ.	Malic acid	Signif. differ.	Acetic acid	Signif. differ.	Wine acid	Signif. differ.	Lactic acid	Signif. differ.
1.	Bogovinje	4.8±0.4	ab	4.9±0.1	a	4.5±0.4	cd	5.6±0.5	ab	6.8±0.6	b
2.	Vrutok	7.0±3.5	efgh	6.8±4.1	bcd	6.5±3.3	abcd	8.2±4.1	cd	9.8±4.9	defg
3.	Dzepchishte	5.5±2.3	bcd	5±1.2	a	5.2±2.1	cde	6.4±2.7	abcd	7.7±3.2	ab
4.	Galate	6.5±1.9	cdef	6.5±2.0	bcd	6.1±1.8	bcd	7.6±2.3	cdef	9.1±2.7	cdef
5.	Zelino	6.4±1.7	cdef	6±1.8	abc	6±1.6	bcd	7.4±2.0	cdef	8.9±2.4	bcde
6.	Pechkovo	5.4±1.2	bcd	7.7±2.3	cde	5.1±1.1	cde	6.3±1.4	abcd	7.6±1.7	ab
7.	Jegunovce	4.8±0.7	ab	5.7±1.0	ab	4.5±0.7	cd	5.6±0.8	ab	6.7±1.0	b
8.	Avtokomanda	5.8±2.3	bcde	6.6±2.2	bcd	5.4±2.1	cdef	6.8±2.7	bcde	8.1±3.2	abc
9.	Sopishte	5.5±3.9	bcd	6.1±4.3	abc	5.2±3.7	cde	6.5±4.6	abcd	7.8±5.5	ab
10.	Drachevo	5.9±1.9	bcde	7.4±1.9	abcde	5.6±1.8	cdef	6.9±2.3	bcde	8.3±2.7	abc
11.	Saraj	6.4±0.9	cdef	7.1±0.7	abcd	6±0.8	bcd	7.5±1.0	cdef	9±1.2	bcde
12.	Radishani	7.3±1.0	efgh	7.8±0.9	cde	6.8±1.0	cdefg	8.6±1.2	cd	10.3±1.5	e
13.	Vlae	5.6±0.8	bcd	6.1±1.4	abc	5.2±0.8	cde	6.5±1.0	abcd	7.8±1.2	ab
14.	Glumovo	6.6±0.6	cdef	7±0.7	abcd	6.2±0.6	bcd	7.8±0.7	cdef	9.3±0.9	bcde
15.	Cheshinovo	5.2±1.3	bcd	5±1.0	a	4.9±1.2	cde	6.1±1.5	abc	7.4±1.9	ab
16.	Karbinci	11.5±8.1	h	8.1±5.4	g	10.8±7.6	g	13.5±9.5	d	16.2±1.4	a
17.	Obleshevo	6.5±1.3	cdef	6.9±1.4	abcd	6.1±1.2	bcd	7.7±1.5	cdef	9.2±18	bcde
18.	Lozovo	7.1±0.8	efgh	7.3±0.8	abcd	6.6±0.7	abcd	8.3±0.9	cd	9.9±1.1	defg
19.	Mustafino	6.7±0.7	cdef	6.8±0.6	bcd	6.3±0.6	abcd	7.9±0.8	cd	9.5±0.9	defgh

The values in each column marked with the same letters do not differ significantly for p <0.05 according to the Duncan test.

(Radishani). In the second harvest, total acidity was higher in the Skopje region, locality Radishani and lower in the Tetovo region (Vrutok). In the third harvest, as in the first harvest, total acidity was higher in the Ovche Pole region, the location of Karbinci, while the lowest concentration was measured in the Tetovo region, Jegunovce location (Table 8).

According to the results of measurements in

Table 7. Partial correlation coefficients for organic acids in relation to measuring points for third harvest.

Control variables			Citric acid	Malic acid	Acetic acid	Wine acid	Lactic acid	Total acidity
Region	Citric acid	Correlation	1	**-0.294**	**1**	**1**	**1**	0.982
		Significance (2-tailed)		0.024	0	0	0	0
				**	**	**	**	**
		df	0	57	57	57	57	57
	Malic acid	Correlation	**-0.294**	1	**-0.294**	**-0.294**	**-0.294**	-0.116
		Significance (2-tailed)	0.024		0.024	0.024	0.024	0.381
			**		**	**	**	**
		df	57	0	57	57	57	57
	Acetic acid	Correlation	**1**	**-0.294**	**1**	**1**	**1**	0.982
		Significance (2-tailed)	0	0.024		0	0	0
			**	**		**	**	**
		df	57	57	0	57	57	57
	Wine acid	Correlation	**1**	**-0.294**	**1**	1	**1**	0.982
		Significance (2-tailed)	0	0.024	0		0	0
			**	**	**		**	**
		df	57	57	57	0	57	57
	Lactic acid	Correlation	**1**	**-0.294**	**1**	**1**	1	0.982
		Significance (2-tailed)	0	0.024	0	0		0
			**	**	**	**		**
		df	57	57	57	57	0	57
	Total acidity	Correlation	0.982	-0.116	0.982	0.982	0.982	1
		Significance (2-tailed)	0	0.381	0	0	0	
			**	**	**	**	**	
		df	57	57	57	57	57	0

** Significantly for p <0.05 according to the Duncan test
Correlation strength: 0-0.50 weak; 0.51-0.75 medium and> 0.75 strong.

Table 9, by harvests and regions, only the total acidity in the third harvest showed a significant difference (p = 0.035 <0.05 for α = 0.01) (α is the coefficient of separation of the Duncun test) between the regions and the total acidity for all three harvests together showed a significant difference (p=0.024 <0.05 for α = 0.01) between the regions.

From Table 10, it can be seen that all investigated organic acids are in a strong positive correlation between themselves (r =1.000, p =0.000 <0.05) except malic, Acid that is in a weakly significant negative correlation (r = -0.071, p = 0.346 > 0.05). The concentration of total acidity is the result of the concentration of organic acids, which depends on several factors. The contents of organic acids and the total acidity in cultural and medicinal plants is correlated with the distance to the pollution source. At low distances, the concentrations of organic acids are generally higher.

CONCLUSIONS

Based on our results, we can draw the following conclusions:

- Organic acids are key metabolites in plants, participate in the synthesis of proteins, carbohydrates and fats and serve as photosynthetic and respiratory intermediates or as metabolic intermediates;
- Based on the analysis of organic acids and of total acidity, there are significant differences among the different locations and regions within the territory of the Republic of North Macedonia.

Regarding the investigated concentrations of organic acids and their total acidity, in all three harvests and in the three regions studied, the concentration for each acid was measured individually, and it can be concluded that

Table 8. Total acidity in alfalfa (*Medicago sativa* L.) from the study sites in the territory of the Republic of North Macedonia in the first, second and third harvest, expressed in % (DM).

Number	Location	First harvest	Signif. differ.	Second harvest	Signif. differ.	Third harvest	Signif. differ.
1.	Bogovinje	29.1 ± 3.9	abc	39.8 ± 7.6	bcdef	28 ± 2.6	ab
2.	Vrutok	40.7 ± 3.7	f	26 ± 5.8	bc	38.3 ± 14.9	bcdef
3.	Dzepchishte	30.2 ± 6.1	bcd	36 ± 7.0	cdef	29.8 ± 9.4	bcd
4.	Galate	30.6 ± 7.2	bcd	28.2 ± 4.5	bcd	35.7 ± 8.4	bcde
5.	Zelino	33.4 ± 7.6	cdef	35.1 ± 4.8	cdef	34.7 ± 8.0	bcde
6.	Pechkovo	27.4 ± 3.3	abc	38.1 ± 2.2	bcdef	32.2 ± 6.6	abcd
7.	Jegunovce	36.1 ± 8.1	de	32.2 ± 6.4	abc	27.2 ± 4.0	a
8.	Avtokomanda	26 ± 3.8	ab	26.6 ± 4.2	bc	32.6 ± 10.4	abcd
9.	Sopishte	32.2 ± 3.7	cdef	40.6 ± 2.3	de	31 ± 16.5	abcd
10.	Drachevo	32.1 ± 2.9	cdef	33.8 ± 7.8	abc	34.2 ± 6.9	bcde
11.	Saraj	36.4 ± 6.3	de	35.8 ± 5.9	cdef	36.1 ± 3.3	cde
12.	Radishani	24.5 ± 1.5	ab	43.6 ± 4.9	h	40.7 ± 4.4	fgh
13.	Vlae	30.1 ± 3.6	bcd	40.9 ± 2.2	de	31.2 ± 3.1	abcd
14.	Glumovo	30.3 ± 6.7	bcd	33.1 ± 5.8	abc	36.9 ± 2.5	cde
15.	Cheshinovo	26.7 ± 1.9	abc	26.8 ± 1.8	bc	28.7 ± 6.2	ab
16.	Karbinci	37 ± 7.8	de	37.2 ± 3.6	bcdef	60.1 ± 31.2	g
17.	Obleshevo	40.8 ± 5.3	f	40.3 ± 5.6	de	36.4 ± 5.4	cde
18.	Lozovo	30.5 ± 1.5	bcd	40.7 ± 6.6	de	39.2 ± 3.3	bcdef
19.	Mustafino	33.4 ± 7.8	cdef	34.4 ± 5.1	abc	37.2 ± 2.4	cde

The values in each column marked with the same letters do not differ significantly for $p < 0.05$ according to the Duncan test.

Table 9. Labelling of organic acid and total acidity after a significant difference in the concentration of harvests and regions.

Slopes	Regions	Citric acid	Malic acid	Acetic acid	Wine acid	Lactic acid	Total acidity
1	Tetovo	$5.84^{a,1}$	$6.12^{a,1}$	$5.47^{a,1}$	$6.84^{a,1}$	$8.21^{a,1}$	$32.48^{a,1}$
	Skopje	$5.50^{a,1}$	$5.38^{a,1}$	$5.16^{a,1}$	$6.45^{a,1}$	$7.74^{a,1}$	$30.22^{a,1}$
	Ovche Pole	$6.01^{a,1}$	$6.50^{a,1}$	$5.64^{a,1}$	$7.05^{a,1}$	$8.4^{5a,1}$	$33.65^{a,1}$
2	Tetovo	$6.10^{a,1}$	$6.30^{a,1}$	$5.67^{a,1}$	$7.09^{a,1}$	$8.51^{a,1}$	$33.62^{a,1}$
	Skopje	$6.54^{a,1}$	$6.83^{a,1}$	$6.13^{a,1}$	$7.66^{a,1}$	$9.19^{a,1}$	$36.35^{a,1}$
	Ovche Pole	$6.35^{a,1}$	$7.20^{a,1}$	$5.95^{a,1}$	$7.44^{a,1}$	$8.93^{a,1}$	$35.88^{a,1}$
3	Tetovo	$5.76^{a,1}$	$6.10^{a,1}$	$5.40^{a,1}$	$6.75^{a,1}$	$8.10^{a,1}$	32.30^{a}
	Skopje	$6.16^{a,1}$	$6.87^{a,1}$	$5.77^{a,1}$	$7.22^{a,1}$	$8.66^{a,1}$	34.68^{a}
	Ovche Pole	$7.42^{a,1}$	$6.84^{a,1}$	$6.95^{a,1}$	$8.69^{a,1}$	$10.43^{a,1}$	40.32^{a}
All together	Tetovo	$5.88^{a,1}$	$6.17^{a,1}$	$5.51^{a,1}$	$6.90^{a,1}$	$8.27^{a,1}$	32.80^{a}
	Skopje	$6.07^{a,1}$	$6.36^{a,1}$	$5.69^{a,1}$	$7.11^{a,1}$	$8.53^{a,1}$	33.75^{a}
	Ovche Pole	$6.59^{a,1}$	$6.85^{a,1}$	$6.18^{a,1}$	$7.73^{a,1}$	$9.27^{a,1}$	36.62^{a}

The mean values in each column marked with the same letters do not differ significantly for $p < 0.05$ according to the Duncan test.
The mean values in each column marked with the same numbers do not differ significantly for $p < 0.01$ according to the Duncan test.

the highest measured concentration for all acids and their total acidity is in the third slope at the Karbinci location in the Ovche Pole region, and the lowest concentration for all acids, excluding malic is measured in the first harvest at the Radishani site in the Skopje region, and the malic in the second harvest in location Galate in the Tetovo region.

According to the results of measurements by harvests and regions, only the total acidity in the third harvest showed a significant difference between the regions and

Table 10. Partial correlation coefficients for organic acids in relation to regions for all harvests together.

Control variables			Citric acid	Malic acid	Acetic acid	Wine acid	Lactic acid	Total acidity
Region	Citric acid	Correlation	1	-0.071	1	1	1	0.973
		Significance (2-tailed)		0.346	0 **	0 **	0 **	0 **
		df	0	177	177	177	177	177
	Malic acid	Correlation	-0.071	1	-0.071	-0.071	-0.071	0.159
		Significance (2-tailed)	0.346		0.346	0.346	0.346	0.034
		df	177	0	177	177	177	177
	Acetic acid	Correlation	1	-0.071	1	1	1	0.973
		Significance (2-tailed)	0 **	0.346		0 **	0 **	0 **
		df	177	177	0	177	177	177
	Wine acid	Correlation	1	-0.071	1	1	1	0.973
		Significance (2-tailed)	0 **	0.346	0 **		0 **	0 **
		df	177	177	177	0	177	177
	Lactic acid	Correlation	1	-0.071	1	1	1	0.973
		Significance (2-tailed)	0 **	0.346	0 **	0 **		0 **
		df	177	177	177	177	0	177
	Total acidity	Correlation	0.973	0.159	0.973	0.973	0.973	1
		Significance (2-tailed)	0 **	0.034 **	0 **	0 **	0 **	
		df	177	177	177	177	177	0

** Significantly for p <0.05 according to the Duncan test.
Correlation strength: 0-0.50 weak; 0.51-0.75 medium and> 0.75 strong.

the total acidity for all three harvests together showed a significant difference between the regions.

All investigated organic acids have a strong positive correlation between themselves (citric, acetic, wine and lactic) with the exception of malic acid. The malic acid is in a weak, non-significant negative correlation with citric, acetic, wine and lactic acid.

The fact that plants synthesize a large number of different organic acids indicates their importance in the metabolism of plants. The amount of synthesised organic acids depends on respiration, enzymatic reactions as well as on growth and development processes, which are impacted by various environmental factors.

All this suggests that alfalfa can be reliably used as a supplement in human nutrition because of the high concentration of organic acids.

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